TOTAL COLIFORM VERIFICATION PROCEDURE
AND
FECAL COLIFORM VERIFICATION PROCEDURE

Colony verifications are extremely important for several reasons. Verification data provide useful support of the laboratory=s findings in any legal action or compliance issue. Also, the verification procedure is beneficial in evaluating an analyst=s ability to accurately identify typical coliform colonies. In addition, the verification procedure helps to classify colonies which are difficult to identify visually.

Because of the membrane filter (MF) procedure used in the detection and enumeration of total and fecal coliform bacteria is strictly a visual identification, subject to analyst interpretation, a confirmatory technique is sometimes required. The procedure given below, referred to as the verified test, should be performed when suspected total coliforms are detected in potable water samples, or at least monthly as a quality control practice, to verify the results of membrane filtrations and/or to maintain familiarity with the test procedure. Laboratories having more than one analyst involved with microbiological analyses must have each analyst verify colonies on a plate at least every three months. The verification test is especially important when mixed growth (colonies with different morphological characteristics) is present or when atypical coliform colonies are being recovered.

I. Total Coliform Colony Verification and Subsequent Fecal Coliform Determination

Under the Safe Drinking Water Act, waters producing total coliform colonies using M-Endo media must be verified. Those colonies which verify positive must be further assessed for the presence of fecal coliform bacteria or E. coli.

The Total Coliform verification test utilizes the fermentation responses for Lauryl Tryptose Broth (LTB) and Brilliant Green Lactose Bile (BGLB) Broth to differentiate total coliforms from background microorganisms that may be recovered using the MF technique. EC medium is used to verify colonies which are fecal coliform. The media used in this procedure are similar to the media required in the MPN (Most Probable Number) test. However, the procedures are not equivalent. In this test, the fermentation reactions are used to demonstrate that the typical red with metallic-green sheen colonies presumed to be total coliform organisms will, in fact, produce the positive (gas) responses under specified conditions. Gas accumulation in the inverted Durham tube indicates a positive response.

The verification procedure is a two-step process. The first step, called the **presumptive** test, is an enrichment technique which allows the bacteria culture to grow actively after being stressed. The LTB tubes are inoculated with a single transfer of inoculum from an isolated colony of a specific morphological type. For quality control purposes, at least five typical red with metallic-green sheen colonies and atypical colonies representative of each morphological group identified (at least one colony of each morphological type or color) must be selected for verification. The tubes are then incubated at 35^{o} " 0.5^{o} C and checked for gas formation after 24 hours. Positive tubes are transferred to EC and BGLB media with a sterile 3 mm inoculating loop or wooden applicator stick. Negative tubes are returned to the incubator for an additional 24 hours. After the second incubation period, positive tubes (gas) are transferred to EC and BGLB medium and all remaining tubes (no growth/no gas and/or growth/no gas) are considered negative (non-coliforms).

The second step of the verification procedure is called the **confirmed** test. After inoculum from the positive LTB tubes have been transferred to tubes of EC and BGLB media, the tubes are incubated. Tubes of BGLB medium are incubated at 35 " 0.5E C for 24 to 48 hours. The BGLB tubes have 24 - 48 hours to produce a positive fermentation (gas) response. If no gas production is noticed after 24 hours, the tubes must be incubated for an additional 24 hours (48 hours total). All BGLB tubes not producing a positive (gas) response within 48 hours are considered to be of non-coliform origin. Tubes of EC medium are incubated at 44.5 " 0.2E C for 24 hours. Tubes of EC medium must not be incubated longer than 24 hours. Gas production indicates that fecal coliform are present. All tubes of EC medium not producing a positive (gas) response within 24 hours are considered not to be fecal coliform.

Procedure:

1) Sterilize a 3 mm inoculating loop using a Bunsen burner, propane torch, or an alcohol lamp. A wood applicator stick, sterilized by dry heat, can also be used. If an alcohol lamp is used, heat the loop to glowing, as alcohol can leave a residue on the inoculating loops and needles. Air cool the inoculation loop before making transfers.

2) Using a wide-field dissecting microscope (10 - 15X) and fluorescent light source to view the bacteria colonies, pick a small amount of growth from the center of an isolated colony grown on medium. A typical total coliform will be red and have a metallic-green sheen.

3) Transfer the colony to a fermentation tube of Lauryl Tryptose Broth by submersing the loop/stick at least one inch below the surface of the medium and gently shaking.

4) Incubate the LTB cultures at 35^{o} " 0.5^{o} C for 24 hours and transfer all gas-positive tubes to EC and BGLB media, inoculating the tube of EC medium first. Re-incubate all LTB negative tubes for an additional 24 hours, then transfer inoculum from all of the 48-hour gas-positive LTB tubes to tubes of EC and BGLB media. All LTB tubes which fail to produce a positive (gas) response within 48 hours are considered non-coliforms.

5) The transfers to the EC and BGLB media are also made with a sterile 3 mm loop or a wooden applicator stick. Inspect each transfer to ensure that a thin film of liquid culture or inoculum is adhering to the loop/stick.

6) Incubate the BGLB tubes at 35.0 " 0.5^{o}C for 24 hours. If gas is not present, incubate for an additional 24 hours. Gas-positive tubes are considered total coliforms and gas-negative tubes are considered to be of non-coliform origin.

   Incubate the EC tubes at 44.5 " 0.5E C for 24 hours. Gas-positive tubes are considered fecal coliform. If EC tubes do not produce gas after 24 hours, **do not** incubate for an additional period of time.

   NOTE: It is permissible to inoculate tubes of LTB, BGLB, and EC media at the same time with inoculum from a single isolated colony in an effort to reduce the time of the analysis. However, due to the selective properties of the BGLB medium, the LTB and EC medium must be inoculated before the BGLB. Also, if all tubes are inoculated at the same time, but only the LTB tube produces gas after the incubation period, fresh tubes of EC and BGLB must be inoculated from the LTB and incubated for an additional 24-48 hours.

II. Fecal Coliform Verification

Laboratory analyst must verify fecal coliform colonies grown on a plate using M-FC medium. Enumeration of fecal coliforms is allowed for source water under the Safe Drinking Water Act. Also, wastewater effluents are tested for fecal coliform bacteria as part of NPDES permit requirements. Because a fecal coliform count is required for these tests, colonies suspected of being fecal coliform must be verified individually.

The Fecal Coliform verification test utilizes the fermentation responses for Lauryl Tryptose Broth (LTB) and EC medium to differentiate fecal coliforms from background microorganisms that may be recovered using the MF Technique. EC medium is used to verify colonies which are fecal coliform. The media used in this procedure are similar to the media required in the MPN (Most Probable Number) test. However, the procedures are not equivalent. In this test, the fermentation reactions are used to demonstrate that the typical blue colonies presumed to be fecal coliform organisms will, in fact, produce the positive (gas) responses under specified conditions. Gas accumulation in the inverted Durham tube indicates a positive response.

The verification procedure is a two-step process. The first step, called the **presumptive** test, is an enrichment technique which allows the bacteria culture to grow actively after being stressed. The LTB tubes are inoculated with a single transfer of inoculum from an isolated colony of a specific morphological type. For quality control purposes, at least five typical blue colonies and atypical colonies representative of each morphological group identified (at least one colony of each morphological type or color) must be selected for verification. The tubes are then incubated at 35° " 0.5° C and checked for gas formation after 24 hours. Positive tubes are transferred to EC medium with a sterile 3 mm inoculating loop or wooden applicator stick. Negative tubes are returned to the incubator for an additional 24 hours. After the second incubation period, positive LTB tubes (gas) are transferred to EC medium, and all remaining tubes (no growth/no gas and/or growth/no gas) are considered negative (non-coliforms).

The second step of the verification procedure is called the **confirmed** test. After inoculum from the positive LTB tubes have been transferred to tubes of EC medium, the tubes are incubated. Tubes of EC medium are incubated at 44.5 " 0.2E C for 24 hours. Tubes of EC medium must not be incubated longer than 24 hours. Gas production indicates that fecal coliform are present. All tubes of EC medium not producing a positive (gas) response within 24 hours are considered not to be fecal coliform.

Procedure:

1) Sterilize a 3 mm inoculating loop using a Bunsen burner, propane torch, or an alcohol lamp. A wood applicator stick, sterilized by dry heat, can also be used. If an alcohol lamp is used, heat the loop to glowing, as alcohol can leave a residue on the inoculating loops and needles. Air cool the inoculation loop before making transfers.

2) Using a wide-field dissecting microscope (10 - 15X) and fluorescent light source to view the bacteria colonies, pick a small amount of growth from the center of an isolated colony grown on medium. A typical total coliform will be light to medium blue.

3) Transfer the colony to a fermentation tube of Lauryl Tryptose Broth by submersing the loop/stick at least one inch below the surface of the medium and gently shaking.

4) Incubate the LTB cultures at 35° " 0.5°C for 24 hours and transfer all gas-positive tubes to EC medium. Re-incubate all LTB negative tubes for an additional 24 hours, then transfer inoculum from all of the 48-hour gas-positive LTB tubes to tubes of EC medium. All LTB tubes which fail to produce a positive (gas) response within 48 hours are considered non-coliforms.

5) The transfers to the EC medium are also made with a sterile 3 mm loop or a wooden applicator stick. Inspect each transfer to ensure that a thin film of liquid culture or inoculum is adhering to the loop/stick.

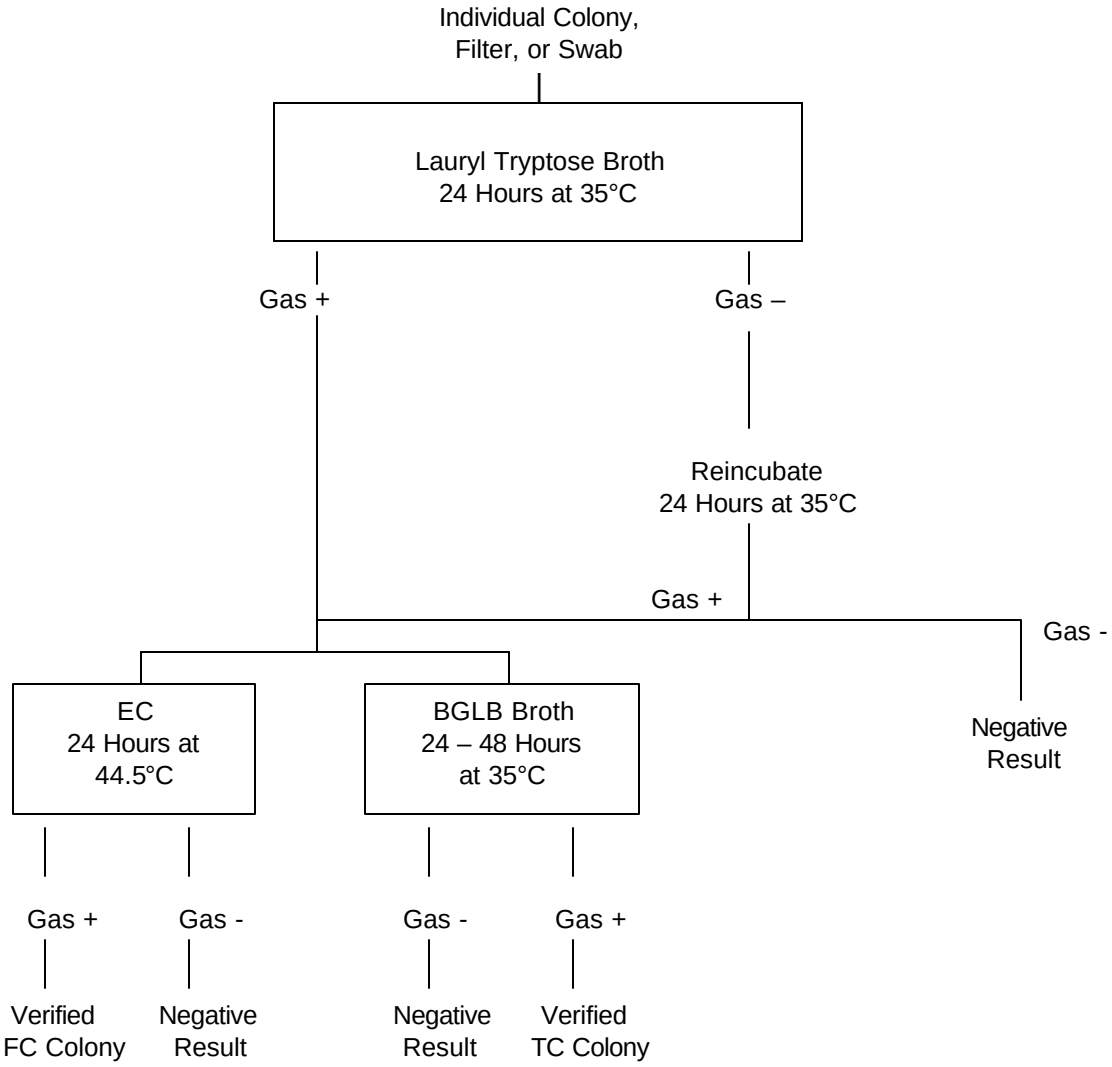
6) Incubate the EC tubes at 44.5 " 0.5E C for 24 hours. Gas-positive tubes are considered fecal coliform. If EC tubes do not produce gas after 24 hours, **do not** incubate for an additional period of time.

NOTE: It is permissible to inoculate tubes of LTB and EC media at the same time with inoculum from a single isolated colony in an effort to reduce the time of the analysis. If both tubes are inoculated at the same time, but only the LTB tube produces gas after the incubation period, fresh tubes of EC medium must be inoculated from the LTB and incubated for an additional 24 hours.

Please be reminded that proper temperature control is essential to the success of the fecal coliform confirmation procedure. For this reason, the level of water in the water bath must extend beyond the level of the media in the EC tubes by at least one quarter (**3**) inch. It is also imperative that once inoculated, the EC tubes are placed in the water bath within 30 minutes to discourage the growth of any coliforms of nonfecal origin and/or background microorganisms that may be present.

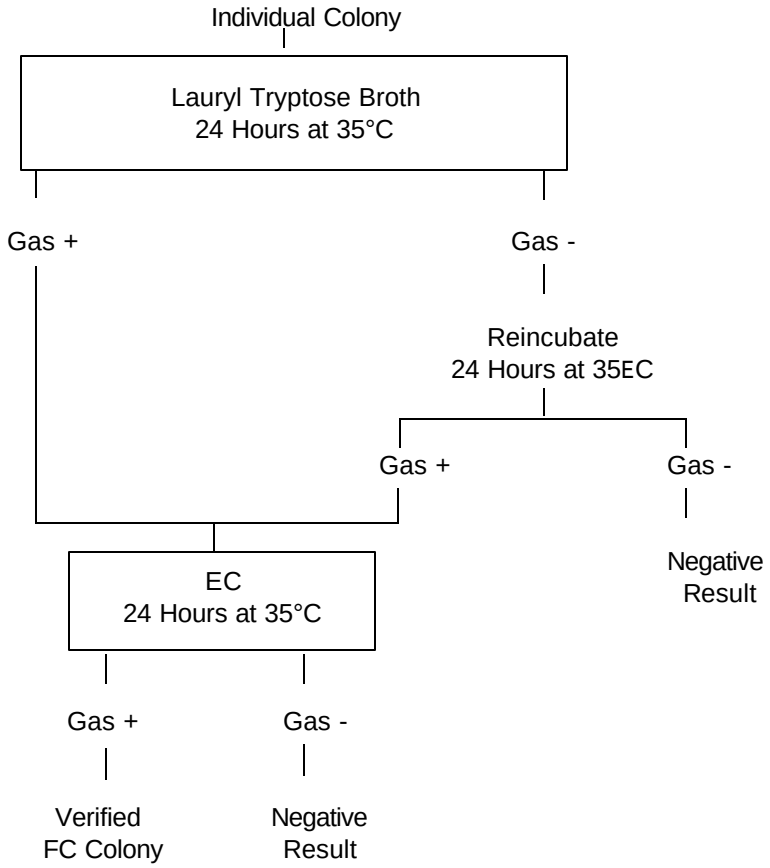
SUSPECTED TOTAL COLIFORM COLONY VERIFICATION
TYPICAL: RED WITH METALLIC GREEN SHEEN COLONIES

For quality control purposes, pick at least five typical colonies and at least one colony from each morphological type identified from a single membrane filter.



SUSPECTED FECAL COLIFORM COLONY VERIFICATION
TYPICAL: BLUE COLONIES

For quality control purposes, pick at least five typical colonies and at least one colony from each morphological type identified from a single membrane filter.



III. Adjusted Colony Counts

During the colony verification process, the analyst may learn that some colonies that have been classified as atypical may indeed be total or fecal coliform bacteria. In these cases, the atypical colonies should be counted as such. As stated earlier, the analyst is required to select (at random) at least five typical colonies and at least one colony from each atypical morphological type. Note that the analyst should verify more colonies of a morphological type if there is an abundance of these colonies. Also note that adjusted counts can only be done on plates from which colonies have been verified.

To perform an adjusted count, determine the percent of colonies verified as total coliforms or E. coli for each morphological type (typical and atypical). Use this percent figure to adjust the reported coliform count per 100 ml. Calculate this by dividing the number of colonies which produce positive results by the number ot colonies tested for each type. Multiply this value by the total number of colonies of that type.

For example, a plate may have 30 Ared with green sheen colonies@ 15 atypical Apink@ colonies, and 10 atypical Abrown@ colonies. All five Ared with green sheen colonies@ verified produce gas in LTB and BGLB, while two of the three Apink@ colonies verified produce gas in LTB and BGLB. Neither of the brown colonies produced gas in BGLB. The final adjusted count would be

Description	+ Colonies/# Tested		# of Colonies of Each Type		Adjusted Number
Red with Green Sheen	5/5	X	30	=	30
Pink	2/3	X	15	=	10
Brown	0/2	X	10	=	0
Total Adjusted Count					40

Although it is required that the analyst, at a minimum, perform colony verifications at least once each month, it is the responsibility of the analyst to perform verifications more frequently if previous verifications show that Atypical@ colonies are not total coliforms and/or that Aatypical@ colonies produce gas in LTB, BGLB, and EC media.

EXAMPLE: WATER BACTERIOLOGY - VERIFIED TEST ON MEMBRANE FILTER TOTAL COLIFORM COLONIES WITH FECAL COLIFORM CONFIRMATION, ADJUSTED COUNTS

DESCRIPTION OF GROWTH ON M-Endo PLATE	LAURYL TRYPTOSE OR LACTOSE		BGLB BROTH		EC MEDIUM
	24 HR.	48 HR.	24 HR.	48 HR.	24 HR.
55 Total Colonies					
30 Typical Red/Sheen					
Typ #1	-	+	+		+
Typ #2	+		+		+
Typ #3	+		+		+
Typ #4	-	+	-	+	-
Typ #5	+		-	+	-
15 Atypical Pink					
Pink #1	-	-			
Pink #2	-	+	+		+
Pink #3	+		-	+	-
10 Atypical Brown					
Bro #1	+		-	-	
Bro #2	-	-			
Colony Count Adjustments:					
Typical 5/5 x 30	= 30				
Pink 2/3 x 15	= 10				
Brown 0/2 x 10	= 0				
Total Coliform Colony Adjusted Count	40				

EXAMPLE: WATER BACTERIOLOGY - VERIFIED TEST ON MEMBRANE FILTER FECAL COLIFORM COLONIES WITH FECAL COLIFORM CONFIRMATION, ADJUSTED COUNTS

	DESCRIPTION OF GROWTH ON MFC PLATE	LAURYL TRYPTOSE OR LACTOSE		EC BROTH
		24 HR.	48 HR.	24 HR.
55 Total Colonies				
30 Typical Blue	Typical Blue			
15 Cream	Blue #1	-	+	+
10 Pink	Blue #2	-	+	+
	Blue #3	+		+
	Blue #4	-	+	-
	Blue #5	+		+
	Cream			
	Cream #1	-	+	+
	Cream #2	+		-
	Cream #3	-	-	
	Pink			
	Pink #1	+		-
	Pink #2	-	+	-
Colony Count Adjustments:				
Typicals 4/5 x 30	positive in EC Broth =			24
Cream 1/3 x 15	positive in EC Broth =			5
Pinks 0/2 x 10	positive in EC Broth =			+ 0
	Fecal Coliform Colony	Adjusted	Count	29